

Article type: Note

New dot-blot method for evaluating the effect of inactivators on mite and Japanese cedar pollen allergens

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ABSTRACT

As a method of evaluating the effect of inactivators on allergens while suppressing the effect of inactivator on the assay, we developed new dot-blot method which combines immunostaining and protein detection methods. This method is useful for evaluating whether the inactivator can inactivate allergens rather than removing them from the assay.

Keywords: Dot-blot, Cry j 1, Der f 1

Two-site mAb-based sandwich ELISA is the gold standard for allergen analysis (Chapman, Vailes and Ichikawa 2000). Generally, sandwich ELISA is used to measure the amount of the allergen in a solution after the reaction between the inactivator and the allergen (Chapman, Vailes and Ichikawa 2000), as a means to evaluate the potential of the inactivators. However, the inactivators affect the assay's ability to measure allergen levels. Allergen levels measured using ELISA are underestimated due to the effect of the inactivator, thus leading to an overestimation of the inactivation potential. The mAb-based sandwich ELISA may not be a suitable method to screen a large number of the inactivators as candidates, as inactivators that interfere with this assay need to be removed using methods such as dialysis (Lombardero *et al.* 2000).

In this study, to evaluate inactivators while avoiding their interference with the assay's ability, a dot-blot method was used. The dot-blot method is a technique similar to western blotting, which is used for the immunological identification of proteins. Using this method, allergen proteins are spotted directly onto the membrane and the specific antibodies react with the allergen. To evaluate the effect of the inactivators using the dot-blot method, allergens were immobilized on the membrane, the inactivator was treated against the immobilized allergen, followed by detection of the allergens with specific mAbs (Figure 1). A second allergen-carrying membrane was prepared with the same number of immobilized allergens. After inactivator treatment, the allergens were detected with a protein stain, in order to confirm the presence of the allergen released from the membrane due to the effect of the inactivator.

A flow chart of dot-blot method is shown in Figure 1. First, polyvinylidene

di-fluoride (PVDF) membranes (GE Healthcare, Chicago, USA) were hydrophilized with methanol. Then, 2 μ L of diluted Der f 1 (Indoor Biotechnologies, Virginia, USA) as mite allergen or Cry j 1 (Hayashibara, Okayama, Japan) (Yasueda *et al.* 1983) as Japanese cedar pollen allergen were spotted onto two PVDF membranes, and washed three times with PBS.

Membranes were incubated with 2 mL of each inactivator, 0.5 to 1000 ppm sodium hypochlorite (Fujifilm Wako Pure Chemical Corporation, Osaka, Japan), 6 M guanidine (Fujifilm Wako Pure Chemical Corporation), and 3.5 mM sodium lauryl sulfate (SDS) (Nacalai Tesque, Kyoto, Japan) at room temperature for 2 h and washed three times with shaking in PBS. Each membrane was assessed with either immunostaining for specific allergens or gold staining for protein detection.

For immunostaining, the membrane was blocked with PBS and 1% gelatin in cold water fish skin (Sigma-Aldrich, St. Louis, USA), at 37 °C for 10 min, followed by incubation with anti-Der f 1 mAb (5F2.1.8, ITEA, Tokyo, Japan) or anti-Cry j 1 mAb (S10, ITEA) (Sakaguchi *et al.* 1997) at room temperature for 30 min with shaking. The membrane was incubated with horseradish peroxidase (HRP)-conjugated anti-mouse IgG (Invitrogen, Waltham, USA), diluted to 1:1000 in PBS-0.5% Tween 20 (T) and 1% bovine serum albumin (BSA) (Sigma-Aldrich) at room temperature for 30 min with shaking and washed four times with shaking in PBS-T. The membrane was then incubated with 3,3',5,5'-tetramethylbenzidine (Ez West Blue substrate, Atto, Tokyo, Japan) at room temperature for 10 min. Finally, the membrane was washed with a large amount of distilled water.

For protein detection, gold staining (Gold protein detection kit for membranes, Cytodiagnosics, Burlington, Canada) was performed. The membrane was washed with shaking in washing buffer for 10 min, then incubated with 2 mL of gold solution at room temperature for 1 h with shaking. Finally, the membrane was washed twice with shaking in washing buffer and a large amount of distilled water.

Figure 1 shows the evaluation procedure with various inactivators using immunostaining and protein staining. Immobilized Cry j 1 allergens on the membrane were treated with 0.5 to 1000 ppm sodium hypochlorite (Figure 2). Anti-Cry j 1 IgG showed poor reactivity to Cry j 1 on membrane treated with 0.5 to 1000 ppm sodium hypochlorite. In protein staining, pollen proteins treated with 5 ppm sodium hypochlorite remained on the membrane; however, those treated with 50 ppm or more were detached. This result indicates that pollen protein on the membrane was shed due to the high sodium hypochlorite concentration. When performing this method, it is necessary to confirm the presence of the proteins on the membrane with protein staining.

Immobilized Cry j 1 allergens on the membrane were treated with each inactivator (Figure 3a). Anti-Cry j 1 IgG reactivity to allergens on the membrane was first determined using the immunostaining method. Anti-Cry j 1 IgG was reactive under the following conditions: 4 ng of Cry j 1 with PBS (no inactivator) treatment, and 20 ng of Cry j 1 with guanidine and SDS treatment, no Cry j 1 with sodium hypochlorite treatment. Protein staining was then performed to confirm whether Cry j 1 had detached from the membrane due to the inactivator. A minimum of 4 ng of allergen proteins in all

membranes were detected using protein staining and it confirmed the attachment of allergen proteins.

Similarly, immobilized Der f 1 allergens on the membrane were treated with each inactivator (Figure 3b). Anti-Der f 1 IgG was reactive under the following conditions: 4 ng of Der f 1 with PBS (no inactivator), guanidine and SDS treatment, and 20 ng of Der f 1 with sodium hypochlorite treatment. However, allergen detection appeared to be less with guanidine and SDS treatments than with PBS treatment. In protein staining, Der f 1 treated with all inactivator remained on the membrane.

In previous studies involving dot-blot methods, denatured allergens were blotted onto a membrane, and the allergen activity was examined with specific antibodies (Liu *et al.* 2010; Aoki *et al.* 2009). Where inactivated allergen could not be successfully blotted, it was considered that allergen activity was not sufficiently evaluated. In this study, allergen inactivators were accurately evaluated using the dot-blot method, which combines immunostaining and protein detection methods. However, there are some potential improvements to this method. First, it should be noted that this result merely indicates that the epitope for the antibodies which used in this study has lost reactivity. As an improvement method, it would be possible to measure more practical allergen inactivation using sera from allergic patients instead of monoclonal antibodies in this method. In addition, the use of crude antigen instead of purified antigen will lead to more realistic evaluation of allergen inactivation. Furthermore, it is considered that the color intensity of each dot can be quantitatively evaluated by using image analysis software. Alternatively, it may be possible to perform immunostaining and protein

staining with a single membrane by using fluorescent coloring together.

Acknowledgements

This work was funded by ITEA Inc.

Data availability

All data generated or analyzed during this study are included in this article. Further enquiries can be directed to the corresponding author.

Author contribution

All authors were involved in study design and data interpretation. All authors critically revised the report, commented on drafts of the manuscript, and approved the final report.

Funding statement

This work was funded by ITEA Inc.

Disclosure statement

MY and KK are employees of ITEA. MS received research grants from ITEA. KM, TN, and JU have no conflicts of interest.

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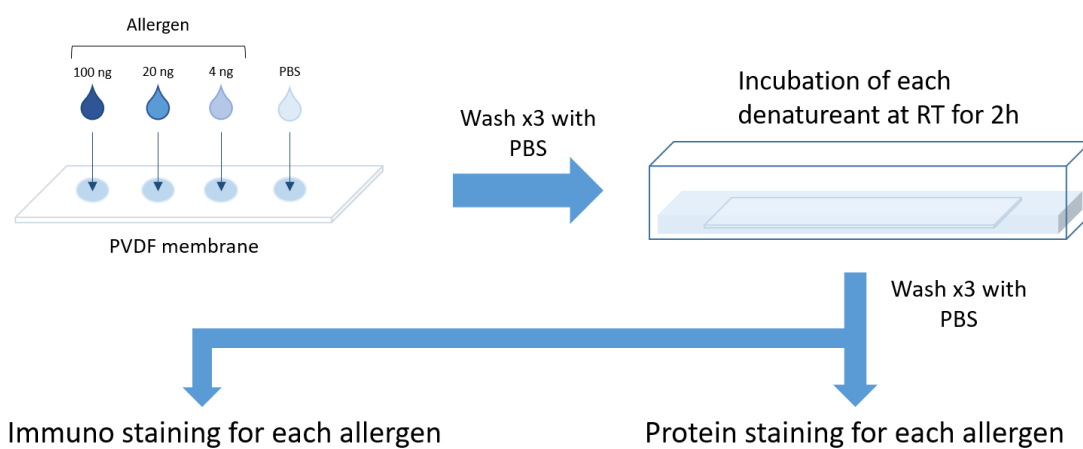


Figure 1. Procedure for evaluation of allergen inactivators using this the dot-blot method that combines immunostaining and protein staining for allergens.

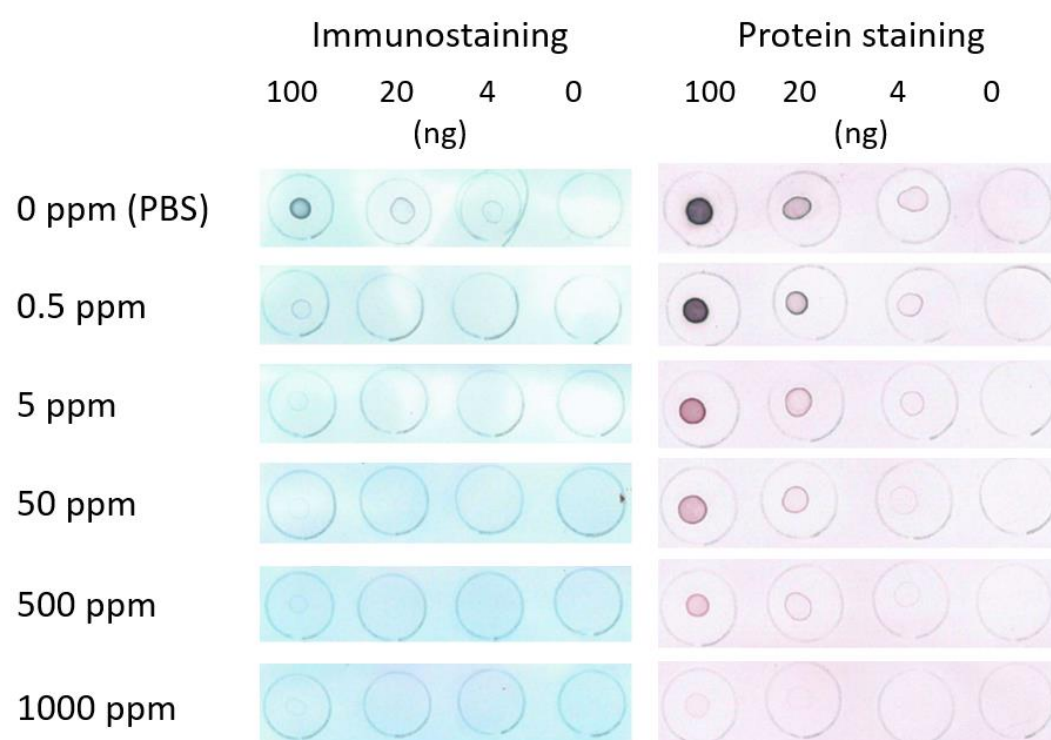


Figure 2. Cry j 1 allergens on the membrane treated with 0.5 to 1000 ppm sodium hypochlorite using the dot-blot method.

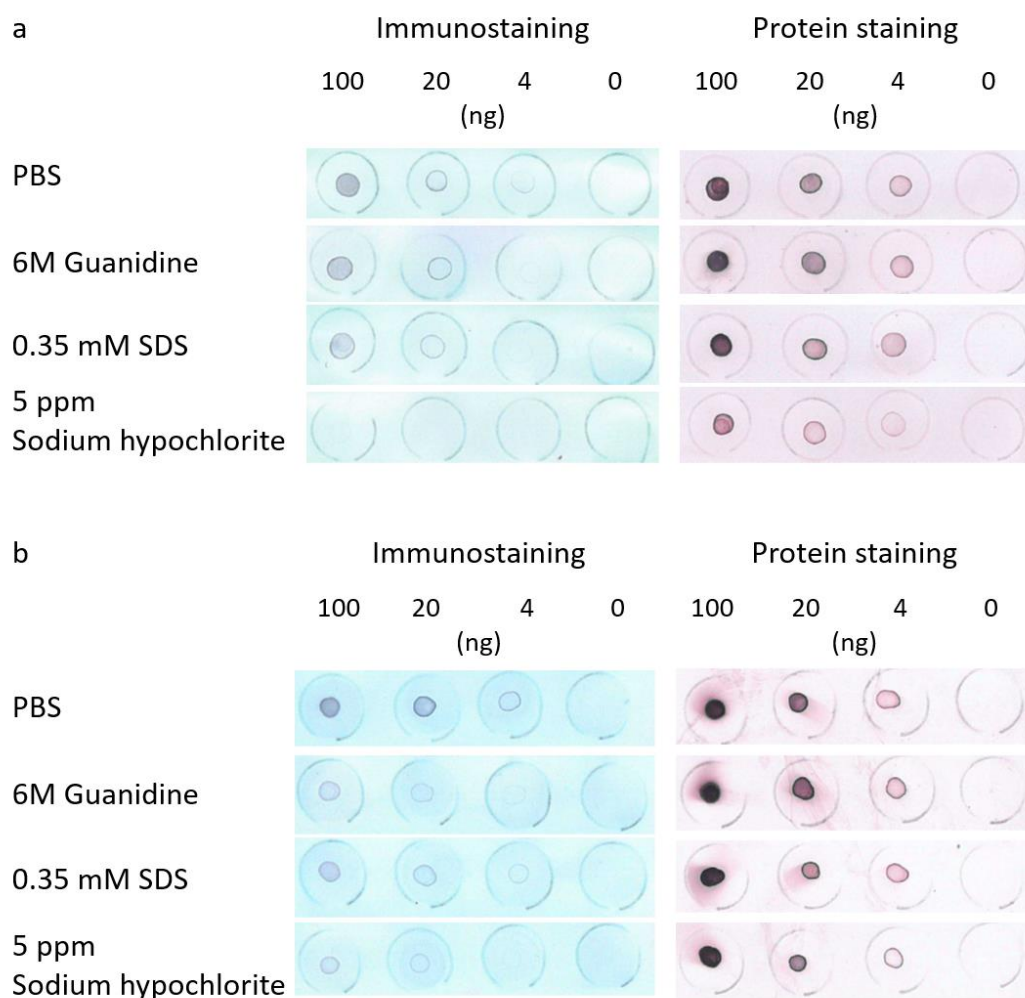


Figure 3. Effects of inactivators on mite and pollen allergens using the dot-blot method.

(a) Cry j 1 (b) Der f 1